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Crimean-Congo Hemorrhagic Fever Virus Genomics and Global Diversity†

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Crimean-Congo hemorrhagic fever (CCHF) is a severe illness with high case fatality that occurs in Africa, Europe, the Middle East, and Asia. The complete genomes of 13 geographically and temporally diverse virus strains were determined, and CCHF viruses were found to be highly variable with 20 and 8%, 31 and 27%, and 22 and 10% nucleotide and deduced amino acid differences detected among virus S (nucleocapsid), M (glycoprotein), and L (polymerase) genome segments, respectively. Distinct geographic lineages exist, but with multiple exceptions indicative of long-distance virus movement. Discrepancies among the virus S, M, and L phylogenetic tree topologies document multiple RNA segment reassortment events. An analysis of individual segment datasets suggests genetic recombination also occurs. For an arthropod-borne virus, the genomic plasticity of CCHF virus is surprisingly high.

Crimean-Congo hemorrhagic fever (CCHF) virus, genus *Nairovirus* and family *Bunyaviridae*, occurs from Sub-Saharan Africa to western China, reflecting the broad distribution of *Hyalomma* ticks, the predominant vector (32, 44, 46, 51, 53). Human infections occur through tick bites, direct contact with blood or tissue of infected livestock, or nosocomial infections and can result in severe hemorrhagic fever with case fatalities of ca. 30% (22, 46, 48). The virus RNA genome consists of the small (S), medium (M), and large (L) segments which encode the viral nucleocapsid (N), glycoprotein precursor (GPC), and polymerase (L) proteins, respectively (41). Complete nucleotide sequences of 25 S, 20 M, and only 4 L segments of different CCHF virus strains (including complete genomes for only two strains) have been determined previously (see Table S1 in the supplemental material). Previous phylogenetic analyses were restricted to partial S segment sequences or limited numbers of complete S and M segments (5, 9, 17, 18, 24, 31, 32, 35, 37, 52). To thoroughly examine CCHF virus genome diversity, evolution, and geographic distribution we successfully developed an approach to rapidly and accurately generate an additional 13 complete virus genome sequences from geographically diverse strains of CCHF virus collected over approximately 50 years (Table 1). The collective comparison of the 15 CCHF complete virus genomes and the sequences of individual complete 32 S, 32 M, and 18 L RNA segments allowed detailed insight into CCHF virus evolution and distribution.

Features of virus RNA segments and encoded proteins. RNA viruses generally have high rates of accumulation of mutations due to the error-prone nature of their polymerases (19). However, arthropod-borne RNA viruses often show remarkably low levels of genome diversity. A popular explanation for this is the double-filter concept, by which it is thought that arbovirus evolution is severely constrained by their having to maintain high fitness in both arthropod and amplifying vertebrate host environments (50). Based on this concept, it was striking to find a high level of CCHF virus genome plasticity. Although overall genome RNA segment and open reading frame (ORF) lengths (Table 1) and important motifs were well conserved, nucleotide variations of 20, 31, and 22% for the S, M, and L RNA segments and amino acid variation of 8, 27, and 10% for the N, GPC, and L proteins, respectively, were found, suggesting that considerable genome and protein diversity can be tolerated while maintaining high fitness in the diverse environments of tick and vertebrate amplifying hosts. Alternatively, the double filter may not operate on CCHF virus if virus fitness in vertebrate hosts is not critical to virus maintenance due to the high efficiency virus transovarial and transtadial transmission within ticks.

As expected, the greatest accumulation of mutations was seen in the surface glycoprotein encoding M RNA segment (31% nucleotide and 27% amino acid divergence). This may reflect varying positive selection operating in the form of immune selection or selection for efficient attachment to different combinations of arthropod and vertebrate host cells in different natural cycles throughout the virus geographic range. The virus exists across numerous ecologic zones, with different *Hyalomma* species tick vectors important in different regions (6, 46 and 53). Differences in tick feeding preferences and vertebrate host availability in the various regions will likely mold the evolutionary landscape of the virus.

Alternatively, lack of constraint (rather than positive selection) may also be making a considerable contribution to the amino acid high diversity observed, since much of the amino acid variation resided in the hypervariable mucin-like domain close to the amino terminus of the M encoded protein (39). This and similar virus-encoded mucin-like domains, such as those found in Ebola glycoproteins (45, 43), are rich in serine, threonine, and proline amino acids; heavily O glycosylated; and highly variable. It appears that maintenance of high levels

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TABLE 1. CCHF virus strains with complete genomes sequenced

Virus strain[a]	Country of origin	Source of isolate[b]	Isolation yr	Passage history[c]	Genome segment features[d]					
					S segment		M segment		L segment	
					Total length (nt)	Major ORF	Total length (nt)	Major ORF	Total length (nt)	Major ORF
AP92	Greece	*R. bursa*	1975	SMB+7, E6+1	1,659	56-1504	5,416	78-5165	12,170	78-11915
ArD8194	Senegal	*H. truncatum*	1969	SMB?	1,686	56-1504	5,386	78-5180	12,160	77-11914
ArD15786	Senegal	Goat	1972	SMB?	1,686	56-1504	5,386	78-5180	12,160	77-11914
ArD39554	Mauritania	*H. marginatum*	1984	SMB?	1,673	56-1504	5,431	78-5165	12,160	77-11914
C-68031	China	Sheep	1968	SMB+20	1,672	56-1504	5,367	78-5147	12,168	78-11915
Drosdov	Russia: Astr.	Human	1967	SMB+27	1,671	54-1502	5,364	78-5144	12,151	79-11916
IbAr10200*	Nigeria	*H. excavatum*	1966	E6?	1,672	56-1504	5,366	93-5147	12,160	77-11914
Kashmanov	Russia	Human	1967	SMB13	1,673	56-1504	5,364	78-5144	12,149	78-11915
Matin*	Pakistan	Human	1976	E6?	1,671	56-1504	6,367	78-5147	12,168	78-11915
Oman	Oman	Human	1997	E6+1	1,673	56-1504	5,361	78-5141	12,176	78-11915
SPU103/87	South Africa	Human	1987	SMB+2	1,673	56-1504	5,364	93-5147	12,158	77-11914
SPU415/85	South Africa	Human	1985	SMB+4	1,673	56-1504	5,354	78-5147	12,157	77-11914
SPU97/85	South Africa	Human	1985	SMB+4	1,673	56-1504	5,345	78-5147	12,157	77-11914
Turkey200310849	Turkey	Human	2003	E6+1	1,673	56-1504	5,364	78-5144	12,149	78-11915
UG3010	DRC	Human	1956	SMB+13	1,640	56-1504	5,388	81-5180	12,128	78-11915

[a] *, Complete genome sequences were derived prior to this study. In addition, although the S segments of strains AP92, ArD8194, C-68031, Drosdov, SPU415/85, and UG3010 were already in GenBank, they were resequenced here in order to obtain complete genomes from the same viral stocks.

[b] *H.*, *Hyalomma*; *R.*, *Rhipicephalus*.

[c] Virus propagation and passages (+ the number) were performed in a biosafety level four (BSL4) laboratory. ?, Unknown passage number. Virus RNA was extracted from suckling mouse brain (SMB) homogenates from mice that had been virus infected by intracranial inoculation or from infected VeroE6 (E6) cells, and RNA was purified by using an RNaid kit (Qbiogene, Inc., Carlsbad, CA).

[d] Single-step reverse transcription-PCR assays were performed using the SuperScript III One-Step RT-PCR system with Platinum *Taq* High Fidelity (Invitrogen, San Diego, CA) under the following conditions: 52.5°C for 30 min, 94°C for 2 min, and then 40 cycles of 94°C for 15 s, 50.5°C for 30 s, and 68°C for 1 min/kb, with a final extension cycle of 68°C for 5 min. The entire S segment of each virus strain was reverse transcription-PCR amplified using the primers CCHF-SF (5′-TCT CAA AGA AAC ACG TGC CGC-3′) and CCHF-SR (5′-TCT CAA AGA TAT CGT TGC CGC-3′). For most strains, the complete M segment was amplified using the primers CCHF-MF (5′-TCT CAA AGA AAT ACT TGC-3′) and CCHF-MR (5′-TCT CAA AGA TAT AGT GGC-3′). The L segment of most viruses was amplified in two overlapping halves, L1 and L2, using the primers CCHF-L1F (5′-TCT CAA AGA TAT CAA TCC CCC C-3′) and CCHF-L1R (5′-TTG GCA CTA TCT TTC ATT TGA C-3′) for the first half and CCHF-L2F (5′-GAA GAG CTA TAT GAC ATA AGG C-3′) and CCHF-L2R (5′-TCT CAA AGA AAT CGT TCC CCC CAC-3′) for the second half. A few strains required strain-specific M and L amplification primers, which were designed and used as sequences became available. Purified PCR product DNA was directly sequenced. For each segment, all respective GenBank nucleotide sequences were aligned, and sequencing primers representing the most conserved regions were designed encompassing the entire segment (a total of 25 S, 40 M, and 84 L primers). Some additional strain-specific sequencing primers were used as sequences became available. Approximately 200 to 230 reads were obtained for each genome, resulting in a ninefold average redundancy at each base position. Sequences were analyzed and assembled as described earlier (10, 11, 14). This approach allowed the efficient generation of the complete genomes of the 13 diverse virus strains (the GenBank accession numbers for virus L, M, and S segments are DQ211612 to DQ211624, DQ211625 to DQ211637, and DQ211638 to DQ211650, respectively).

of O-glycan addition is the primary constraint relative to function, and considerable amino acid variation is easily tolerated. The function of the CCHF virus mucin-like domain remains to be determined, but by analogy with Ebola virus (another hemorrhagic fever-associated virus) it may play an important role in pathogenesis (45, 43).

Despite the high M ORF diversity observed, the evolution of functional domains important in processing and trafficking of the GPC (3, 15, 29, 39, 40, 49), appeared to be highly constrained. The furin cleavage site (39) is conserved in all strains; the SKI-1 or SKI-1-like protease recognition sites R(R/K)LL located by the Gn N and C termini are completely conserved, as is the R(R/K)PL site located at the N terminus of Gc (49).

Four predicted N glycosylation sites exist in virus strain IbAr10200 Gn (residues 557 and 755) and Gc (1054 and 1563) proteins. Three of these sites (557, 1054, and 1563) are completely conserved in all strains analyzed, and only strains UG3010, ArD8194, and ArD15786 (which are monophyletic on the M tree) lack the potential glycosylation at site 755. Interestingly, recent studies (3, 15) have reported that Gn contains a Golgi retention signal, whereas Gc may have an ER retrieval sequence. Complete conservation of a motif reported to be an ER retrieval motif in retroviral glycoproteins and other cellular proteins (13, 16, 23) was observed, with a histidine and a lysine at positions −3 and −5 within the C termini of mature Gc.

RNA virus polymerase genes are generally considered among the most conserved genes within the segmented RNA viruses. Consequently, it was surprising to find a higher level of L gene and L protein diversity (22 and 10%, respectively) than that observed within S segment and N (20 and 8%, respectively). Consistent with earlier analyses (20, 25, 30), the RNA polymerase core domains (within the region from approximately amino acid 762 onward) were highly conserved among the CCHF viruses, whereas other regions exhibited high diversity, more in keeping with hinge or spacer-type regions seen in other transcription/replication-associated molecules. The large size (relative to many viruses in other genera of the family *Bunyaviridae*) of the CCHF virus L polymerase (almost 4,000 amino acids in length), together with the high conservation of an ovarian tumor-like protease domain (residues 35 to 152) and a zinc finger type C2H2 domain (residues 606 to 632) among all of the CCHF viruses (20, 25), suggests that nairovirus L polyproteins may have functions in addition to the polymerase functions known for other members of the family *Bunyaviridae*. The L protein amino acid diversity and conservation detailed here provide a basis for probing function by

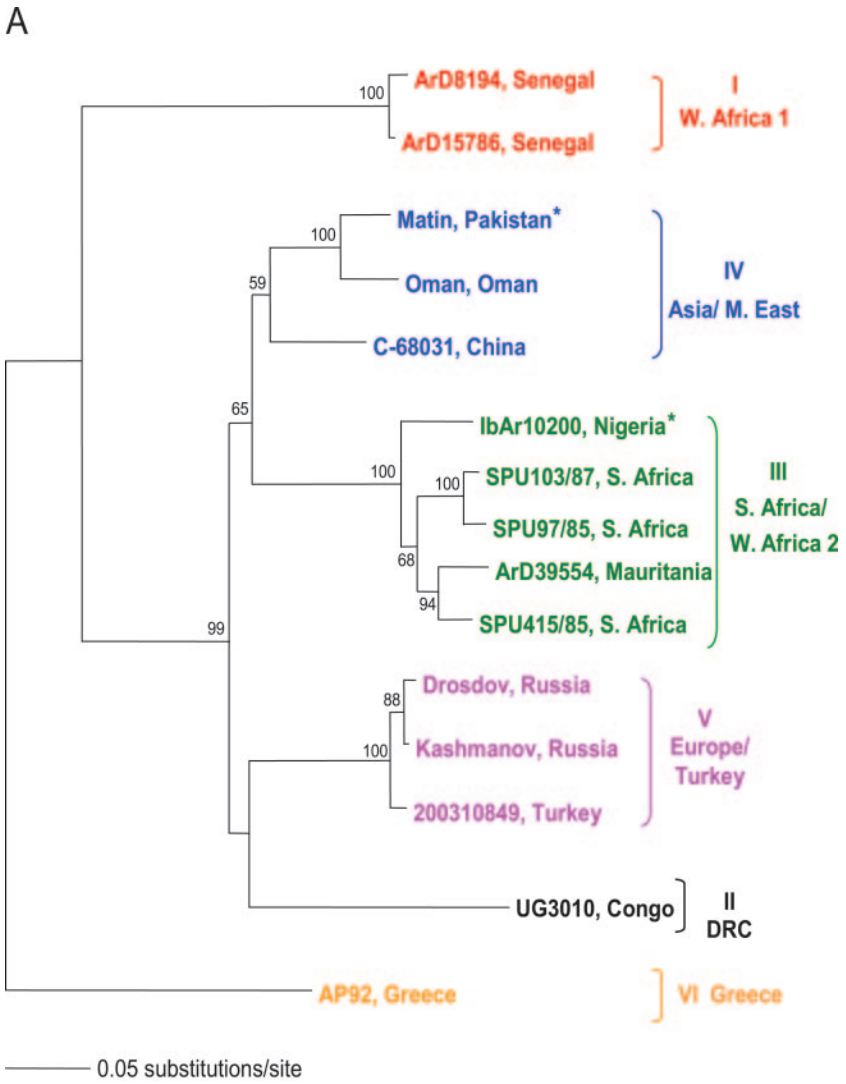

FIG. 1. Phylogenetic relationships of CCHF virus full-length S segments. Phylogenetic analysis was carried out using PAUP version 4.0b10 (Sinauer Associates, Inc., Sunderland, MA). Using maximum-likelihood criteria, the optimal evolutionary model employed utilized a transition/transversion (Ti/Tv) ratio of 5.80, a proportion of invariable sites of 0.29, and a γ distribution value of 0.5. Bootstrap values were calculated from analysis of 500 pseudoreplicates of the data set, and values greater than 50% are indicated at the appropriate nodes. Each virus sequence is designated by the name of the strain and its country of origin. Brackets indicate the virus genetic groupings. In addition, the different virus groups are color coded for clarity. (A) Phylogenetic tree generated from 15 S segment nucleotide sequences from viruses with complete genomes. The asterisk indicates the two strains that were not sequenced during this study within the 15 sets. (B) Phylogenetic tree constructed from all 32 complete S segment nucleotides available.

means of mutational analysis and reverse genetics methods currently under development.

Phylogenetic evidence of virus geographic movement. The collective phylogenetic comparison of the 15 CCHF complete virus genomes and the sequences of individual complete 32 S, 32 M, and 18 L RNA segments revealed seven distinct virus groups and their approximate geographic distribution (Fig. 1, 2, and 3). The tree branching patterns were robust, with high bootstrap values obtained for most nodes. In addition, tree topologies based on nucleotide differences were virtually identical to those based on amino acid differences (data not shown). The groupings included: group I, West Africa 1; II, Democratic Republic of the Congo (DRC); III, South Africa and West Africa 2; IV, Asia and the Middle East, V, Europe and Turkey, VI, Greece; and VII, Mauritania (detected only with the M segment). These groupings demonstrate that specific CCHF virus lineages move over large geographic distances. For example, closely related viruses can be found in South Africa and West Africa or in Iraq and China. Multiple virus genetic lineages can also be found in some geographic areas (e.g., group I and III viruses in West Africa). Several explanations of these phylogeographic features are possible. Movement of CCHF virus-infected livestock (or uninfected livestock carrying infected ticks) via trade may explain some of the movement of virus genetic lineages within regions. For instance, there is considerable movement of sheep and goats into the Arabian Peninsula from countries in the horn of Africa or Iran and Pakistan, particularly in association with major

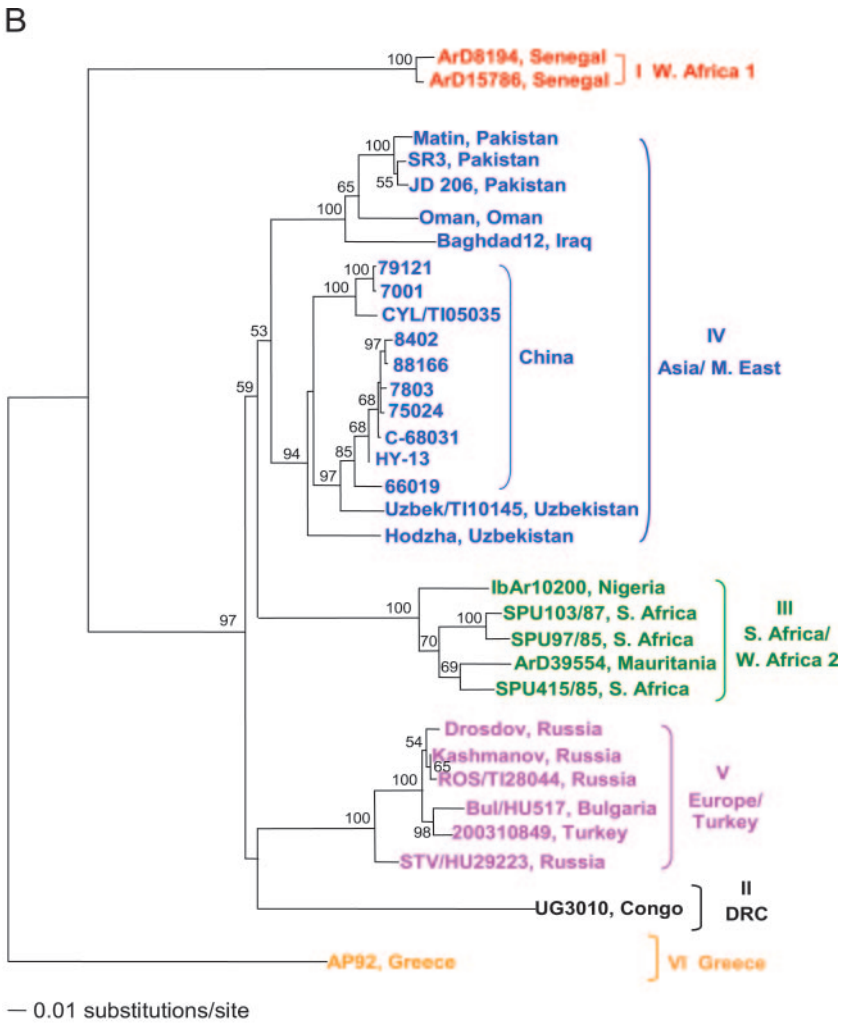

FIG. 1—*Continued.*

religious festivals. The genetic links seen between virus strains and detailed epidemiologic and genetic analysis of past CCHF virus outbreaks in United Arab Emirates and Oman are consistent with this view (37).

Movement of genetic lineages of CCHF virus, particularly over greater distances and between regions not linked by livestock trade, likely also involves migratory animals or birds that are either infected or are carrying virus-infected ticks (21, 22, 48). Although some studies have suggested birds are not readily infected with CCHF virus, ostriches and several West African ground-feeding birds have been shown to be susceptible to infection, and even refractory species could move attached infected ticks without themselves becoming infected (22, 42, 47, 54, 55). Examination of major migratory bird flyways suggests this type of movement could provide a plausible explanation for virus lineage linkages between such areas as West and South Africa, for instance.

RNA segment reassortment. Although mutation is the primary means of increasing RNA virus genome diversity, RNA reassortment can also play an important role in the segmented RNA viruses, with the most dramatic example being the influenza A viruses, where segment reassortment can result in antigenic shift and the emergence of pandemic virus strains (26). Earlier analyses of natural and experimental infections have documented RNA segment reassortment among arthropod-borne viruses of the family *Bunyaviridae* (2, 4, 12, 33, 36, 38). Consistent with earlier analysis of more limited data sets (7), several examples of RNA segment reassortment were revealed by detailed analysis of incongruencies between the more comprehensive S, M, and L data sets presented here (Fig. 1, 2, and 3). Reassortment appears to be much more frequently observed among CCHF virus M segments than among S and L segments.

Essentially identical S and L tree topologies were seen using the segments from 15 complete genome data set (Fig. 1A and 3A), and the same topologies were seen when the analyses were expanded to include the 32 S and 18 L complete segment sequences available (Fig. 1B and 3B). An earlier report (7) had suggested that CCHF virus L and S segment tree topologies were analogous, but the more comprehensive analysis presented here shows that, although they are highly similar, some differences indicative of segment reassortment events do exist.

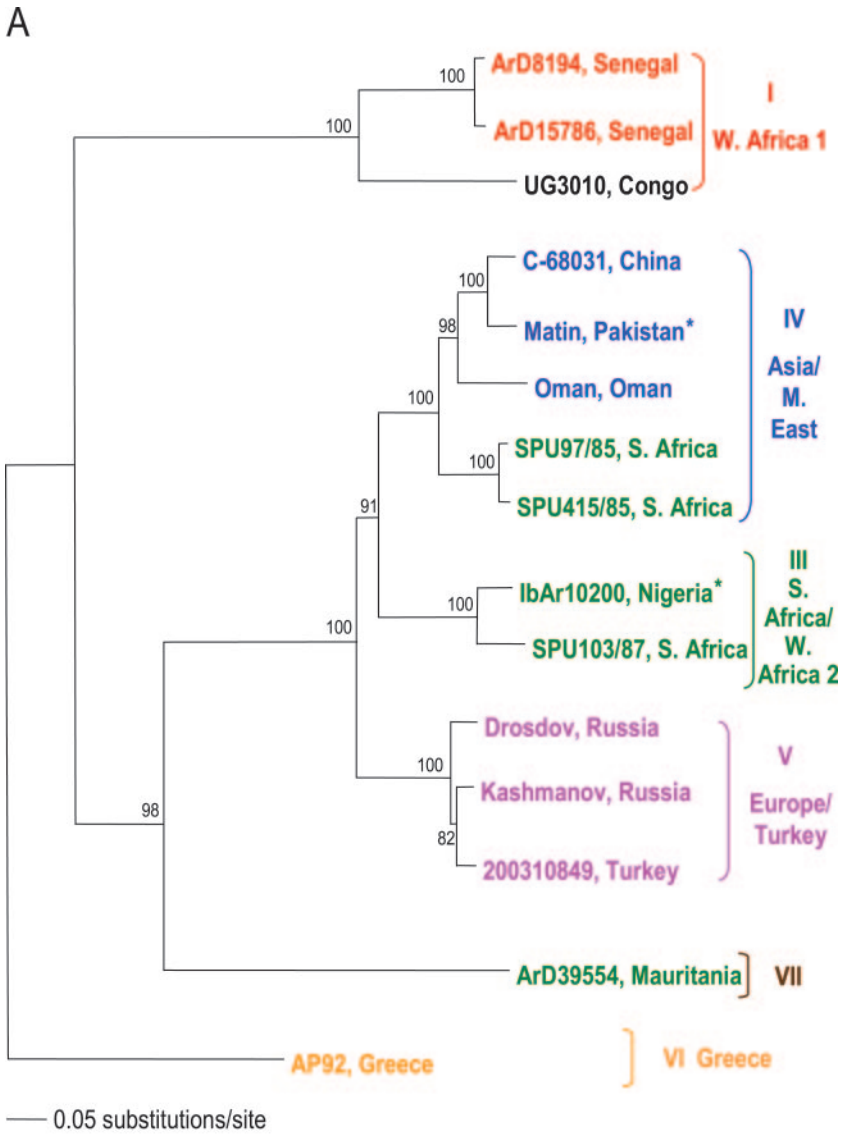

FIG. 2. Phylogenetic relationships of CCHF virus full-length M segments. Using maximum-likelihood criteria the optimal evolutionary model employed utilized a transition/transversion (Ti/Tv) ratio of 6.05, a proportion of invariable sites of 0.13, and a γ distribution value of 0.5. Bootstrap values were calculated from analysis of 500 pseudoreplicates of the data set, and values greater than 50% are indicated at the appropriate nodes. (A) Phylogenetic tree based on the M segments from the 15 CCHF virus strains with available complete genome sequences. (B) Phylogenetic tree constructed from all 32 complete M segments available.

With regard to groups switching relationships, strains ArD8194 and ArD15786 from Senegal constitute group I (West Africa 1) in S tree analysis but belong to group III (South Africa and West Africa 2) in the L segment analysis. From comparison of the S, M, and L tree topologies for these viruses, the most parsimonious interpretation is that strains ArD8194 and ArD15786 likely represent L segment reassortant viruses, with their L segments sharing ancestral origins with those of the South Africa and West Africa group III viruses. It is also noted that virus groups III and IV form well-supported monophyletic clades based on the S and M segment trees but not in the L tree, which is suggestive of a more ancient L reassortment event. Relationship switches (with high bootstrap support) can also be seen between Oman, Matin (Pakistan), and C-68031 (China) strains within group IV, consistent with segment reassortment within this group and also possible recombination among virus L segments (discussed below).

In contrast to the S and L phylogenetic trees, many more examples of RNA segment reassortment can be seen in the M segment phylogenetic trees. Trees based on M segment nucleotide and deduced amino acid sequence differences were very similar to each other, whether based on the 15 virus strains (with complete genome sequences) or on the 32 available full-length M segments (Fig. 2A and B, respectively). These M segment groupings have some differences relative to those suggested earlier using more limited data sets (1, 18). Surprisingly, in the 32-sequence analysis, the additional Chinese strains 79121 and 7001 grouped within group I (West Africa 1)

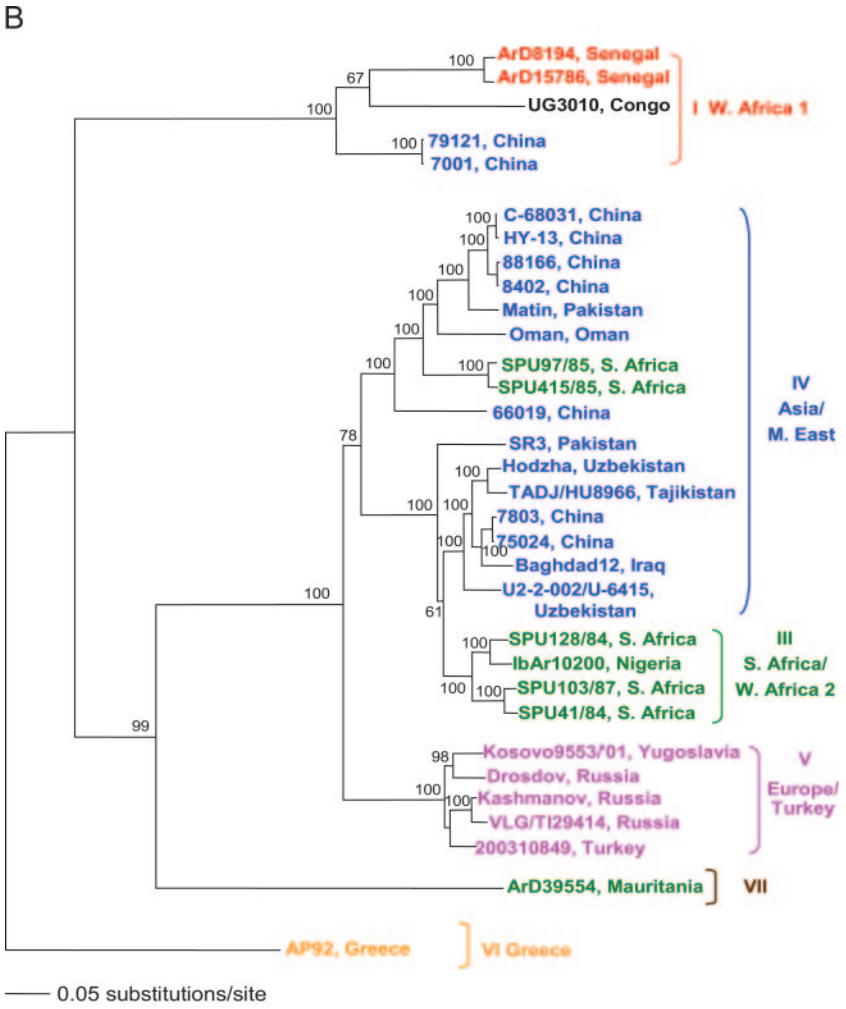

FIG. 2—*Continued.*

with high bootstrap support for this topology (Fig. 2B), suggesting that they representing M segment reassortants. Other potential M segment reassortment events include (i) South African strains SPU415/85 and SPU97/85 that were in group III in both S and L trees now cluster within group IV (Asia and Middle East) in the M segment trees, (ii) Mauritanian strain ArD39554 that belonged to group III in S and L forms a unique group VII in M segment trees, and (iii) Congolese strain UG3010 that formed group V (based on S and L) appears to have acquired a reassortant M segment from group I. Multiple examples of potential M segment reassortments among virus group members were also found. For instance, within group V, the Turkey and Kashmanov strains form a well-supported clade based on M segment data, but strains Kashmanov and Drosdov form a well-supported clade based on S and L datasets (Fig. 1A, 2A, and 3A). Similar instances exist within group III.

These data indicate that CCHF virus M segment reassortment events are more frequent than for S and L segments or more frequently result in high fitness viable virus. Reassortment between viruses from different geographic groups and its dependence on coinfection reinforces the point made above, that movement and mixing of viruses over large geographic distances is occurring with some frequency. It seems likely that genetic reassortment may primarily occur during coinfection of ticks due to the transient nature of vertebrate infections relative to the long-term persistent virus infections seen in ticks and their obligate need to obtain blood meals at metamorphic junctures (34).

Recombination. A low rate of homologous recombination is reported for negative-stranded RNA viruses in general (8). An earlier screening of 17 S and 11 M CCHF virus genome segments found evidence of potential recombination among S but not M segments (8). Similarly, a subsequent study of 18 S, 13 M, and 4 L genome segments revealed strong suggestive evidence of S segment recombination (28). Examination of our more comprehensive S (32), M (32), and L (18) data sets by similarity plot, bootscanning, and analysis of informative sites (27) revealed no convincing evidence of recombination events among M and L segments, with the possible exception of the mosaic nature of L for the group IV viruses (see Fig. S1 in the supplemental material). In agreement with the two earlier studies, evidence of phylogenetic incongruence was found upon analysis of different regions of the S RNA segments of

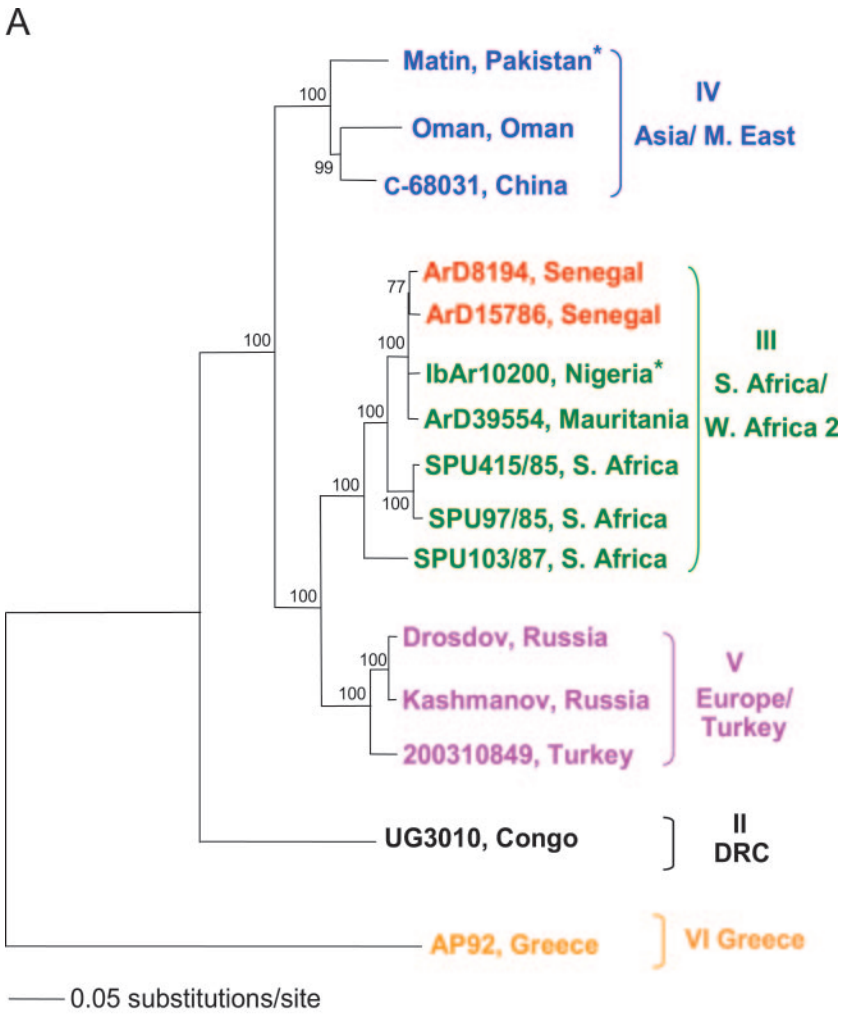

FIG. 3. Phylogenetic relationships of CCHF virus full-length L segments. Using maximum-likelihood criteria the optimal evolutionary model employed utilized a transition/transversion (Ti/Tv) ratio of 8.94, a proportion of invariable sites of 0. 37, and a γ distribution value of 0.5. Bootstrap values were calculated from analysis of 500 pseudoreplicates of the data set, and values greater than 50% are indicated at the appropriate nodes. (A) Phylogenetic tree constructed based on L nucleotide sequences from the same 15 CCHF virus strains analyzed in Fig. 1 and 2. (B) Phylogenetic tree constructed from all 18 complete L segments available.

various CCHF virus strains analyzed here, including the Kashmanov, Drosdov, and STV/HU29223 strains from Russia; Uzbek/TI10145 from Uzbekistan; 66019 and HY-13 from China; and JD206 from Pakistan (data not shown). However, these recombination events involved relatively short genome regions. Collectively, these data suggest that although RNA recombination is relatively rare, it may also contribute, along with accumulation of mutations and segment reassortment, to the high genetic plasticity of CCHF virus.

CCHF virus is on the select agent list of agents considered to have bioterrorism potential due its aerosol infectivity, its ability to cause HF outbreaks with high case fatality (ca. 30%), and its association with nosocomial infections. The comprehensive CCHF virus genomics analysis presented here provides important insight into the evolution and surprisingly high diversity of this virus. In addition, the study provides the highly valuable foundation for the design of molecular detection and characterization tools for the epidemiologic and forensic analysis of outbreaks of both natural and potential deliberate origin.

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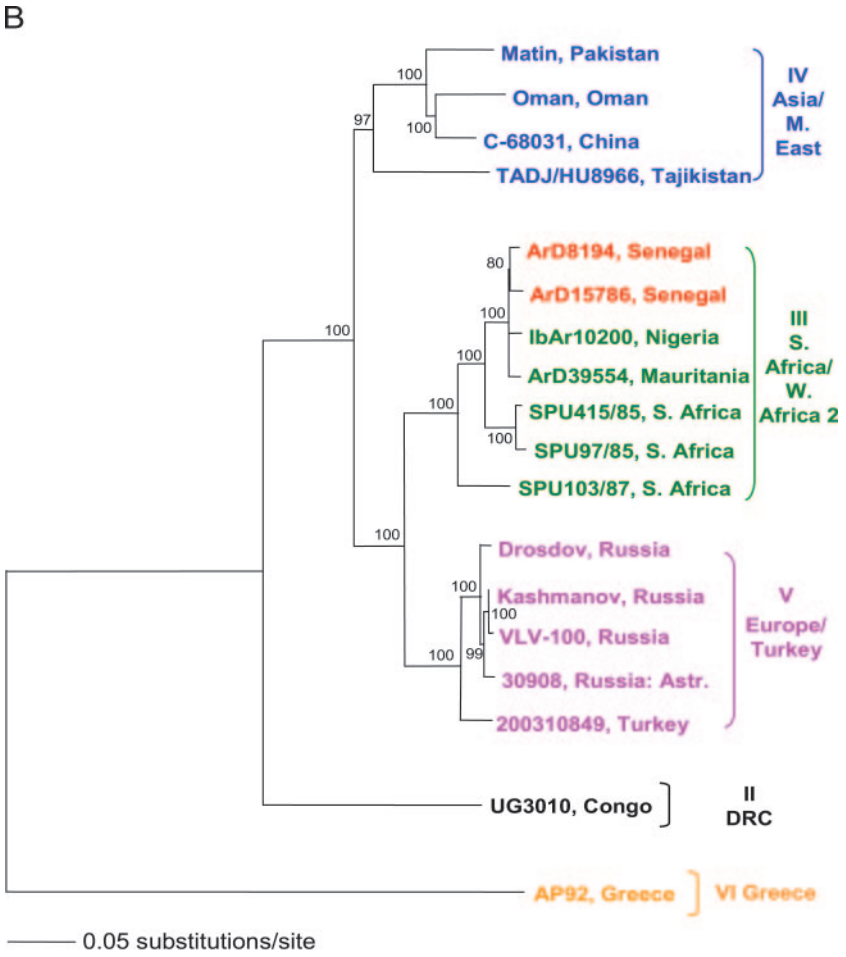

FIG. 3—*Continued.*

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